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Running title: Alpha-enolase as a new PDAC immunotherapeutic target

Key words: human; pancreatic ductal adenocarcinoma; alpha-enolase; tumor antigen; B cell response; T cell response.

Abbreviations:

Ab, antibody; APC, antigen presenting cells; CP, chronic pancreatitis; CTL, cytotoxic T lymphocytes; DC, dendritic cells; 2-DE, 2-dimensional electrophoresis; FCS, fetal calf serum; HLA, human leukocyte antigen; IFN-γ, interferon-γ; IgG, immunoglobulin G; IL, interleukin; mAb, monoclonal antibody; MHC, major histocompatibility complex; NP, normal pancreas; PBMC, peripheral blood mononuclear cells; PDAC, pancreatic ductal adenocarcinoma; PPD, tuberculin purified protein derivative; TAA, tumor associated antigen; Th, T helper cells; WB, western blot.

Abstract

Pancreatic ductal adenocarcinoma (PDAC) is a fatal disease with a very poor 5-year survival rate. Alpha-enolase is a glycolytic enzyme that also acts as a surface plasminogen receptor. We find that it is over-expressed in PDAC and present on the cell surface of PDAC cell lines. The clinical correlation of its expression with tumor status has been reported for lung and hepatocellular carcinoma. We have previously demonstrated that sera from PDAC patients contain IgG autoantibodies to α-enolase. The present work was intended to assess the ability of α-enolase to induce antigen-specific T cell responses. We show that α-enolase-pulsed dendritic cells (DC) specifically stimulate healthy autologous T cells to proliferate, secrete IFN-γ and lyse PDAC cells but not normal cells. *In vivo*, α-enolase-specific T cells inhibited the growth of PDAC cells in immunodeficient mice. In 8 out of 12 PDAC patients with circulating IgG to α-enolase, the existence of α-enolase-specific T cells was also demonstrated. Taken as a whole, these results indicate that α-enolase elicits a PDAC-specific, integrated humoral and cellular response. It is thus a promising and clinically relevant molecular target candidate for immunotherapeutic approaches as new adjuvants to conventional treatments in pancreatic cancer.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related deaths in Western countries. Despite aggressive surgical and medical management, the mean life expectancy is approximately 15-18 months for patients with local and regional disease, and only 3-6 months for those with metastatic disease [1]. Most patients with advanced disease cannot be operated and pancreatic tumor cells are often resistant to chemotherapy and radiation therapy.

The discovery of human TAA has identified genes relevant in cancer progression and recognized by the host immune system [2]. Despite the results in animal models, most clinical trials with antigen-specific vaccines have so far failed to elicit profound tumor rejection responses. Many vaccines induce an albeit weak humoral and cytolytic immune response, whereas a clinically evident anti-tumor response is rarely achieved [3]. The "elimination phase" of cancer immunosurveillance is, of course, not always successful, and tumors with reduced immunogenicity or with gained suppression functions are thought to emerge from the immune selection pressure and ultimately kill the host [4, 5]. In a spontaneous model of *in situ* PDAC in immune-competent animals, indeed, this phase was nearly nonexistent [6]. Thus, the failure of immunosurveillance may be an early event during PDAC cancerogenesis. In humans, indeed, PDAC is an essentially uniformly fatal disease and, although immunotherapy holds promise as a novel strategy in its management [1], more efficacious means are urgently needed. Recent data suggest that immunomodulating doses of cyclophosphamide given before immunotherapy enhance treatment-induced anti-tumor immune responses [7-9], even in the treatment of very aggressive tumors such as those of the pancreas [10]. In this light, cancer vaccines could be employed in pancreatic cancer as adjuvants to conventional treatments and in the management of minimal residual disease following resection of the primary cancer.

In PDAC, only four antigens, namely MUC-1, K-ras, coactosin-like protein, and mesothelin induce specific responses by both CD4 and CD8 T cells [10-13]. MUC-1 and K-ras also elicit a humoral response and have been used in phase I and I/II clinical trials [11, 14]. Anti-mesothelin agents,

namely recombinant immunotoxin, the chimeric anti-mesothelin mAb and the *Listeria monocytogenes*-mesothelin vaccine are used in clinical practice or about to enter in clinical trials [15]. Selection of an appropriate TAA is crucial for the design of vaccines and an essential prerequisite for induction of a strong immune response. The limited number of PDAC-associated antigens that can be candidates for vaccines, and the persistent lack of effectiveness of vaccine trials have prompted our search for new TAA.

SERological Proteome Analysis (SERPA) [16, 17] with PDAC cells as bait to screen IgG in sera from PDAC patients has shown that they, but not healthy donors or non-PDAC-related tumor patients, display a high frequency of circulating IgG to α-enolase [18]. Its ability to induce both CD4 and CD8-mediated responses in healthy donors and PDAC patients, and T-cell-mediated inhibition of PDAC cell growth in *nu/nu* mice have been evaluated.

As our results demonstrated that α-enolase elicits efficient cellular responses to PDAC cells both *in vivo* and *in vitro*, and that PDAC patients expressing circulating IgG to α-enolase display a T cell response to it, we propose α-enolase as a novel molecular target for active and passive immunotherapies in PDAC.

Material and methods

Cells and Tumor Cell Lines.

The human PDAC cell lines CF-PAC-1 (HLA-$A2^+$, ECACC Ref. No: 91112501), Mia-Pa-Ca-2 (HLA-$A24^+$, ECACC Ref. No: 85062806), PANC-1 (HLA-$A2^+$, ECACC ref. No: 87092802), PaCa44 and K562 cells were cultured in DMEM or RPMI 1640 supplemented with 20 mM glutamine, 10% certified FCS (GIBCO, Invitrogen, San Giuliano Milanese, Milan, Italy), and Gentamycin (40 μg/ml; Shering-Plough, Milan, Italy). All the in vitro cultures were performed at 37°C in a humidified 5% CO_2 atmosphere. Peripheral blood mononuclear cells (PBMC), T cells and dendritic cells (DC) were derived from the peripheral blood of healthy donors from the Blood Bank and cancer patients under an Institutional Review Board-approved protocol and with informed consent. Healthy donor whole blood was assayed for HLA-$A2^+$ expression by flow cytometry with fluorescein thio-isocyanate (FITC)-conjugated mouse anti-human HLA-A2 mAb (BD Biosciences, Milan, Italy).

Human keratinocytes.

Due to the unavailability of normal pancreatic cells that matched with HLA class I of T cells, we used normal keratinocytes HLA-$A2^+$ as control in cytotoxic assays. Human split thickness skin grafts (0.4 mm thick) harvested from informed consent patients undergoing routine reconstructive plastic surgery for various disorders pathologies at the Burn Centre of Turin were mostly taken from the breast and a few from the thigh and abdomen. Grafts were put into sterile boxes in an appropriate medium, namely RPMI 1640 supplemented with penicillyn-streptomycine (240 μg/ml and 200 μg/ml; both from Sigma, Milan, Italy), incubated at 4°C overnight and processed the day after.

Skin keratinocytes were cultivated on a lethally irradiated feeder layer of 3T3-J2 cells, as described previously [19]. In brief, samples were incubated with dispase at 37°C for 3-4 hours. After epidermal separation from the dermal sheet, only the epidermal samples were treated with trypsin (0.05%

trypsin and 0.01% EDTA; Sigma). Cells were plated (1.5 x $10^4/cm^2$) on lethally irradiated (3000 rads of X-radiation) 3T3-J2 cells (2.4 x $10^4/cm^2$) and cultured in 5% CO_2 and humidified atmosphere in DMEM containing FCS (10%), insulin (5 mg/ml), hydrocortisone (0.4 mg/ml), cholera toxin (0.1 nM), triiodothyronine (2 nM), glutamine (4 mM), and penicillin streptomycin (50 IU/ml) (all from Sigma). Epidermal growth factor (10 ng/ml, from PeproTech Inc., Rocky Hill, NJ) was added 3 days after plating. The culture medium was changed every 2-3 days. Undifferentiated keratinocytes from subconfluent cultures [20, 21] from different donors were stained with FITC-conjugated mouse anti-human HLA-A2 mAb to freeze and store only those HLA-$A2^+$.

Differential expression of α-enolase in normal and PDAC tissues:

i) Microarray analysis.

RNA was obtained from 3 chronic pancreatitis (CP), 3 PDAC primary tumors and their adjacent normal pancreas (NP) from the surgery Dept. of University of Verona, 8 PDAC xenografts and one PDAC cell line (PaCa44). Snap-frozen surgical samples were ground on dry ice with a mortar and pestle, suspended in RNeasy Lysis buffer and total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Total RNA was checked for quality using a BioAnalyzer Lab-on-a-Chip system (Agilent Technologies, Santa Clara, CA). cDNA were synthesized from 12.5 μg of total RNA according to the manufacturer's instructions (Affymetrix, Santa Clara, CA). After in vitro transcription, labeling and fragmentation, probes were hybridized to the GeneChip HG-U133A containing 22,283 probesets, corresponding to about 15,000 genes. The chips were washed in a GeneChip Fluidics Station 400 (Affymetrix), and the results were visualized with a Gene Array scanner using Affymetrix software. Array data were normalized and summarized using the RMA method [22], supplied in the *Affy* package (http://bioconductor.org/packages/2.0/bioc/src/contrib/affy_1.14.0.tar.gz) for the R statistical software (http://www.r-project.org). To assess differential expression, the values of intensity, normalized with NP, of two probesets (201231_s_at and 217294_s_at), corresponding to *ENO1*

transcripts, and three probesets (204009_s_at, 204010_s_at and 214352_s_at), corresponding to *Kras* transcripts, were averaged and subjected to linear regression analysis.

ii) Western blot analysis.

Fresh frozen pancreatic tissue from 3 patients with PDAC and paired adjacent NP, obtained from the Regina Elena Cancer Institute (Rome, Italy), and 10^7 of CF-PAC-1, PANC-1 cells and keratinocytes were used for one-dimensional WB analysis. Cold lysis buffer containing 50 mM TRIS/HCl pH 7,4, 150 mM NaCl, 1% NP40, 1% Triton X-100, 1 mM DTT, 10 µl/ml inhibitory cocktail, 1 mM PMSF (all Sigma) and 10 µl/ml nuclease mix (GE Healthcare Bio-Sciences, Milan, Italy) was added to a total of 30-50 mg of fresh-frozen tissue, previously homogenised (T18 basic UltraTurrax, IKA, Wilmington, NC), or to CF-PAC-1, PANC-1 cells and keratinocytes (10^7 cells/ml of cold lysis buffer). After sonication with an ultrasound sonicator (Hielscher UP200S, Hielscher Ultrasonics GmbH, Stuttgart, Germany; 3 × 40 s, amplitude 40 %, cycle 0.5), the mix was centrifuged (13000 rpm/min, 30 min, 4°C). Twenty µg of protein extract, measured with the Bradford assay (Bio-Rad Laboratories, Hercules, CA), were run on a small NuPAGE® Novex® 4-12% Bis-Tris pre-cast gel (Invitrogen), and transferred to a nitrocellulose membrane (GE Healthcare Bio-Sciences). Anti α-enolase (clone 19/12) [23] and HRP-conjugated anti-mouse IgG (Santa Cruz, Celbio, Milan, Italy) Abs were used at 1:1000. Immunodetection was accomplished with ECL PLUS (GE Healthcare Bio-Sciences). The resulting chemifluorescent signals were scanned with "ProXPRESS 2D" (Perkin-Elmer) and recorded in TIFF format. Intensity of reactive lines was quantified, linearized on β-tubulin or actin and expressed as arbitrary units (AU) of normalized signal intensity.

iii) Immunohistochemical analysis.

Immunohistochemical analysis on 10 PDAC tissues and 10 paired adjacent NP was performed with an anti-α-enolase mAb (1:100; clone 19/12) followed by a horseradish peroxidase (HRP)-

conjugated goat anti-mouse IgG (Santa Cruz) and DAB detection kit (Ventana Systems, Tucson, AZ). Samples were fixed with formalin and paraffin-embedded. Slides were incubated with the primary and secondary Ab and developed in a Ventana ES automated stainer. Images were acquired on a Leica DM LA upright microscope equipped with a DC300F camera and were analyzed with IM 50 software (Leica, Heidelberg, Germany).

iv) Alpha-enolase immunostaining.

CF-PAC-1 and Mia-Pa-Ca-2 cells were seeded on cover glasses and allowed to adhere overnight in complete medium. Glasses were washed twice with 1% BSA-0.01% sodium Azide DPBS (all from Sigma) and incubated with the anti-α-enolase mAb (clone 72/1) [23] or an isotype control Ig (Dako, Milan, Italy) for 30 min at 4°C. After two washes, glasses were incubated with a TexasRed conjugated anti-mouse IgG and then with the lypophilic Hoechst 44332 (all from Molecular Probes, Invitrogen,) to stain nuclei. Glasses were mounted on slides with Moviol (Sigma) and observed with a Leica TCS SP2 laser scanning confocal microscope driven by Leica Confocal Software. The Images were acquired at room temperature (RT) with a 63 X /1.32 N.A. PL APO objective at 1024 X 1024 pixel resolution, recorded as TIF files and processed (Adobe Photoshop, Mountain View, CA) to subtract background and enhance lower and middle intensity fluorescence.

Production of recombinant histidin-tagged α-enolase.

The enolase coding sequence (aa 10-434) was obtained by restriction cleavage of the full-length human α-enolase cDNA [24]. The 1.35 kb BglII-XhoI fragment was cloned into the bacterial expression vector pRSETA (Invitrogen) in frame with a poly-histidine coding sequence. The recombinant α-enolase was overexpressed in the *E. Coli* strain BL21(DE3)/pLysS (Invitrogen) upon induction with 1 mM IPTG (Sigma) and purified on Ni_2^+ affinity column (Invitrogen) according to the manufacturer's instructions with the following modifications. Bacterial cells were

harvested from 1 liter culture by centrifugation and pellets were resuspended in 20 ml of native binding buffer (NBB, 20 mM Na_2PO_4 and 500 mM NaCl, pH 7.8) with the addition of lysozyme (100 μg/ml) and sarcosyl 0.7%. The suspension was slowly rocked for 15 min at 4°C. The cell lysate was sonicated on ice with ten 40-second pulses at high intensity, then centrifuged at 10000 rpm for 15 min at 4°C to pellet the insoluble fraction, which was resuspended in 10 ml of guanidinium lysis buffer (NBB containing 6M Guanidine Hydrochloride), enriched with 10 μl/ml proteases inhibitor cocktail, and incubated for 10 min at RT. The lysate was added to the equilibrated column (Ni-NTA agarose column, Invitrogen) and incubated for 30 min at 4°C using gentle rotation. Two washes with NBB and 2 with Native Wash buffer (NWB; NBB at pH 6.0) followed. The elution was performed with 10 ml of elution buffer (NBB pH 6.0 containing 350 mM imidazole). The eluted fractions were dialysed in sterile water, then lyophilised and resuspended in sterile pyrogen-free PBS. Aliquots were stored at -20°C. The endotoxin level was less than 0.03 EU/ml in the Limulus Amebocyte Lysate assay (Pyrogent 0.3 plus; BioWittaker Cambrex Biosciences by Lonza, Milan, Italy).

Generation and pulsing of DC.

Human PBMC were isolated from the venous blood of healthy HLA-$A2^+$ donors by Ficoll-Paque density gradient centrifugation (GE Healthcare Bio-Sciences). Monocytes were enriched with a monocyte isolation kit (Miltenyi Biotech, Calderara di Reno, Bologna, Italy). The preparations were consistently > 90% $CD14^+$ as determined by FACSCalibur (BD Bioscences). To prepare immature DCs, the enriched monocytes were incubated in six-well culture plates ($5x10^6$ cells/3ml/well) in RPMI 1640 medium supplemented with 10% certified FCS and 100 ng/ml of GM-CSF and 50 ng/ml of IL-4 (both PeproTech Inc.) for 6 days as previously described [25]. Immature DCs were resuspended at $1x10^6$ cells/ml and incubated with the recombinant Histidin tagged-α-enolase, human albumin (solution 20% of albumin purified from human plasma, Kedrion, Wien, Austria), recombinant human albumin (purified by *Pichia pastoris;* Sigma) or tuberculin

purified protein derivative (PPD, Statens Serum Institut) overnight. After 24 h cells were harvested for co-cultures with autologous T cells.

In vitro priming of healthy T cells: i) Proliferative response analysis.

Unpulsed, α-enolase-, human albumin- and PPD-DC were seeded at decreasing concentration (10^4 to 2500) in RPMI-10% certified FCS in 96-well microplates. After thawing autologous PBMC, T cells were purified using a nylon wool column (Robbins Scientific Corporation, BioSpace, Sunnyvale, CA) or CD4 and CD8 enriched positively with magnetic beads (Miltenyi Biotech) and added at 10^5 cells/well to DC. Nylon wool purified cells are $CD3^+ > 90\%$; positively enriched $CD4^+ > 85\%$ and $CD8^+ > 92\%$ as determined by FACSCalibur. After 5 days, 1 μCi of [methyl-3H]thymidine (^{3}HTdR, 25 Ci/mmol) (GE Healthcare Bio-Sciences) was added to each well for 18 h. Cells were collected with a CELLharvester (Packard Instruments, Meriden, CT, USA) in UNIfilter plates and ^{3}HTdR uptake was quantitated (TopCount microplates scintillation counter; Packard). All tests were performed in triplicate.

ii) IFN-γ Elispot assay.

Nitrocellulose plates (Millipore, Milan, Italy) were coated with the anti-IFN-γ capture mAb (Endogene, by Tema Ricerche, Bologna, Italy) overnight at 4°C. CF-PAC-1 cells or autologous DCs previously pulsed with 10 μg/ml of recombinant α-enolase or human albumin overnight were mixed with T cells from co-culture with unpulsed DC (referred to as "unstimulated T cells") or α-enolase-DC ("α-enolase-stimulated T cells") at Stimulator:T cell ratio of 1:10, for 20 h at 37°C. The T cells, or purified CD4 and CD8 where indicated, were seeded at 2.5 x 10^4-10^5 cells/well. In a few experiments, stimulator cells were incubated with anti-HLA class I (clone W6.32) or class II (clone AA384 kindly provided by Dr. Ada Funaro, University of Turin) mAb and then added to effector cells. The plates were then washed in PBS plus 0.1% of Tween and incubated with a biotin-conjugated anti-IFN-γ mAb (Endogene) for 2 h at 37°C. After washing, avidin-peroxidase was

added to the plate for 30 min. The reaction was developed using AEC substrate (Sigma), and spots were quantified with the microplate reader along with a computer-assisted image analysis system (AID, Amplifon, Buttigliera, Turin, Italy).

iii) Cytotoxicity assay.

Target cells were labeled with 50 μCi sodium chromate ($Na_2{}^{51}CrO_4$, > 50 Ci/g Cr; Perkin Elmer, Milan, Italy) for 60 min at 37°C, washed three times and plated at 10^3 cells/well in a 96-well U-bottom plate with various numbers of purified CD8 effector cells. The plates were briefly centrifuged and incubated for 4 h at 37°C. All determinations were done in triplicate. Fifty μl of supernatants were harvested on LUMA plates (Packard) and counted on a TopCount microplates scintillation counter. Maximum release was obtained by adding 25 μl of Triton X-100 to the labeled target cells. Spontaneous release was obtained by incubating the labeled cells in the absence of T cells. Percent of specific lysis was calculated from the following formula: % specific lysis = 100 x (experimental release - spontaneous release) / (maximum release - spontaneous release). Due to the difficulty of Cr uptake by keratinocytes, cytotoxic activity by α-enolase-stimulated T cells was evaluated with GranToxiLux kit (OncoImmunin Inc., Gaithersburg, MD, US) by flow cytometry following the manufacturer's instruction. In a few experiments, target cells were incubated with anti-HLA class I or class II mAb and then added to effector cells.

In vivo experiments.

One million CF-PAC-1 cells were injected sub cute in athymic *nu/nu* mice (Harlan-Nossan, San Pietro del Natisone, Italy). Three days later, 3 mice were sacrified to histological analysis of tumor takes and the other ones were injected or not intravenously with 10^7 unstimulated, albumin- or α-enolase stimulated T cells from healthy HLA-$A2^+$ donors. Mice were treated with anti-asialo GM-1 rabbit serum (0.2 ml/mouse of a 1:20 dilution; Cedarlane, by Celbio, Milan, Italy) 1 day before and once a week for four weeks after the tumor challenge to eliminate NK reactivity.

Progressively growing masses with a mean diameter of >2 mm were regarded as tumors. Growth was monitored twice a week until a tumor exceeded a mean diameter of 15 mm, when mice were sacrificed according to accepted animal use protocols.

PDAC patient cellular reactivity to α-enolase.

18 PDAC patients were divided into those that did or did not displayed IgG to α-enolase evaluated by SERPA, performed as previously described [26]. Briefly, CF-PAC-1 protein extract was separated by 2-DE, blotted onto a nitrocellulose membrane and probed with PDAC serum (4 h, 1:200 working dilution) or mouse anti-α-enolase mAb (clone 19/12, for 1 h, 1:1000 working dilution). Membranes were incubated with HRP-conjugated goat anti-human IgG or HRP-conjugated goat anti-mouse IgG (both 1 h, 1:2000 working solution, Santa Cruz) and revealed with ECL PLUS. The resulting chemifluorescent signals were scanned with "ProXPRESS 2D" (PerkinElmer) and recorded in TIFF format. Results were expressed as arbitrary units (AU) calculated as the mean of volume intensity of the six α-enolase isoforms. Sera that displayed a more than 0.5 AU volume intensity were classified as positive. The AU range of six negative patients was from 0.02 to 0.33 (mean 0.2 ± 0.15 SD), whereas the AU range of twelve positive patients was from 1.34 to 17.0 (mean 9.9 ± 6 SD).

Two x 10^5 PBMC/well from PDAC patients were stimulated or not with 10μg/ml recombinant α-enolase in a 96-well plates. T cell cultures were fed every 3-4 days with 10 U/ml of IL-2 (PeproTech Inc., by Tebu-bio), and re-stimulated or not with 10μg/ml recombinant α-enolase on day 11 for 20 h. PBMC recovered were seeded in Elispot plates, previously coated with anti-IFN-γ mAb, and kept in an incubator for a further 20 h. The reaction was developed as previously described.

Statistical analysis.

Student's t-test (GraphPad Prism 4 Software, Inc., San Diego, CA) was used to evaluate the differences in T cell proliferation, IFN-γ secretion (no. of spots), % of specific lysis and in mouse tumor diameter at all time points.

Results

Alpha-enolase is over-expressed at both mRNA and protein levels.

Microarray data generated from pancreatic tissues from 3 paired adjacent PDAC NP, 3 PDAC, 3 CP, PaCa44 PDAC cell line and 8 PDAC transplanted in immmunosuppressed mice (PDAC xenograft) show a significant differential level of α-enolase mRNA expression in tumor tissues compared to normal pancreas or CP (Fig. 1*a*). This inflammatory and not neoplastic lesion is histologically characterized by a strong desmoplastic reaction, similar to PDAC [27], while PDAC xenografts are a purified source of adenocarcinoma cells *in vivo*. To assess differential expression, a linear regression analysis was performed on logarithmic intensity values as a function of the sample class. Compared to NP, the expression of α-enolase was significantly enhanced in PDAC ($P < 0.001$), PaCa44 ($P < 0.001$) and PDAC xenografts ($P < 0.001$), whereas no difference of expression was observed with respect to CP samples ($P = 0.419$). Of note, the α-enolase levels in PDAC were comparable with or higher than those of Kras, an established oncogene mutated in virtually all PDAC cases [14].The differential expression of α-enolase was confirmed at protein level by WB and immunohistochemistry. Total protein extract from 3 bioptic PDAC and their paired adjacent NP was analysed with the mAb to α-enolase, and PDAC displayed higher amounts of α-enolase protein compared to NP (Fig. 1*b*). Furthermore, immunohistochemical analysis of neoplastic and adjacent NP lesions of 10 PDAC patients revealed that in the latter, α-enolase expression was restricted to medium-small ducts whereas the acinar cells were negative. In contrast, PDAC tissues diffusely expressed α-enolase in the cells lining the neoplastic ducts infiltrating the stroma (Fig. 1*c*). Confocal microscopy showed that α-enolase was expressed on the surface of not permeabilized CF-PAC-1 (upper right panel) and Mia-Pa-Ca-2 (lower right panel) cells.

Alpha-enolase-DC activate a specific proliferative response by T cells.

To characterize the immunogenic activity of α-enolase, we first assessed its ability to activate

T cells. Autologous DC, as professional APC, were pulsed with recombinant α-enolase and co-cultured with autologous purified T cells from six healthy donors. Compared to unpulsed DC, those pulsed with α-enolase induced a significant increase of T cell proliferation in all donors (Fig. 2*a*). This response was dose-dependent and specific, since it was not induced by albumin-DC (Fig. 2*b*). To exclude any reactivity due to the different preparation of recombinant α-enolase and plasma purified albumin, we also compared the proliferation induced by plasma and *Pichia pastoris* purified albumin and no significant differences were observed (data not shown). Both purified CD4 and CD8 T cells from four healthy donors displayed significant proliferation in response to α-enolase-DC (Fig. 2*c*). In four independently tested donors, α-enolase always induced proliferation from 25 to 50% lower than that induced by the recall Ag PPD, suggesting that the response to α-enolase is a primary and not a recall response (data not shown).

Alpha-enolase-DC induce specific IFN-γ production by CD4 and CD8 T cells.

T cells from healthy HLA-$A2^+$ donors were co-cultured with autologous unpulsed or α-enolase pulsed DC. After 7 days, IFN-γ production was evaluated by Elispot in response to α-enolase-DC, CF-PAC-1 cells and normal keratinocytes (HLA-$A2^+$). Anti-HLA class I or anti-HLA class II mAb were added to determine the dependence of the IFN-γ production on MHC. Unstimulated T cells displayed a few spots that were not affected by the presence of anti-HLA class I or II mAb (Fig. 3*a*). By contrast, α-enolase-stimulated T cells displayed a significantly increased number of IFN-γ secreting cells in response to either α-enolase-DC or CF-PAC-1, but not to normal keratinocytes (Fig. 3*a*). Both anti-HLA class I and II mAb significantly decreased the number of IFN-γ secreting T cells elicited by α-enolase-DC or CF-PAC-1 (Fig. 3*a*). No IFN-γ secreting cells were observed when T cells were primed by DC pulsed with α-enolase pre-absorbed with anti α-enolase mAb (data not shown).

Both unstimulated CD4 and CD8 T cells from healthy HLA-$A2^+$ donors did not produce IFN-

γ when tested against DC pulsed with α-enolase or albumin. In contrast, an increased number of IFN-γ spots were observed in both α-enolase stimulated CD4 and CD8 T cells in response to DC pulsed with α-enolase but not to DC pulsed with albumin (Fig. 3*b*). Of note, only α-enolase stimulated CD8 T cells were induced to secrete IFN-γ by CF-PAC-1 (Fig. 3*b*).

Alpha-enolase induces anti-tumor cytotoxic effector T cells.

HLA-$A2^+$ T cells from healthy donors were co-cultured with unpulsed or α-enolase-DC for 7 days and their ability to lyse two PDAC cell lines was evaluated in a 4 h Cr release assay. Purified CD8 T cells stimulated with α-enolase-DC lysed HLA-$A2^+$ CF-PAC-1, whereas HLA-$A24^+$ Mia-Pa-Ca-2 or autologous DC were not lysed (Fig. 4*a, b, c*). The NK-sensitive K562 cell line used as negative control of the HLA class I-dependent cytotoxicity was not lysed (Fig. 4*d*). The ability of α-enolase to induce CD8 CTL that lyse CF-PAC-1 cells was confirmed in an additional five HLA $A2^+$ healthy donors (Fig. 4*e*). As a whole, these data clearly indicate that *in vitro* α-enolase induces anti-tumor cytotoxic effector T cells.

Alpha-enolase specific T cells do not kill normal cells.

In considering α-enolase as a potential target for immunotherapy, we addressed the question of whether its expression in normal tissues spares them from α-enolase-specific CTL. The ability of α-enolase stimulated CD8 T cells from healthy HLA $A2^+$ donors to lyse HLA matched normal human keratinocytes was evaluated by GranToxiLux assay. WB analysis indicated that HLA-$A2^+$ CF-PAC-1 and PANC-1 cells express 3-fold more α-enolase than keratinocytes (Fig. 5*b*). Alpha-enolase specific CD8 T cells were more efficient in lysing CF-PAC-1 and PANC-1 than keratinocytes, suggesting a direct correlation between α-enolase expression and specific-CTL lysis (Fig 5*a*). Anti-HLA class I but not class II mAb incubation completely inhibited lysis and thus confirmed the dependence of the cytotoxicity on MHC class I.

Alpha-enolase-specific T cells inhibit the growth of PDAC cells in vivo.

The efficacy of α-enolase stimulated T cells to inhibit the growth of PDAC cells *in vivo* was also evaluated. *Nu/nu* mice were injected sub cute with 10^6 CF-PAC-1 cells and three days later, when the tumor was well established, as evaluated by histological analysis (Fig. 6, insert), were challenged or not with 10^7 HLA $A2^+$ unstimulated, albumin or α-enolase-stimulated T cells. Mice were treated with anti-asialo GM-1 to eliminate their NK reactivity against CF-PAC-1 cells. In all groups CF-PAC-1 cells gave rise to 2 mm diameter tumor masses after 9 days and the i.v. injection of unstimulated or albumin stimulated T cells did not affect tumor growth (Fig. 6). A significant delay in the growth of CF-PAC-1 cells, indeed, was only evident in mice injected with α-enolase stimulated T cells (Fig. 6).

T cell responses against α-enolase correlate with the presence of anti-α-enolase IgG in PDAC patients.

As previous data have indicated that anti-α-enolase IgG are present with high frequency in sera from PDAC patients [18], we evaluated whether this humoral response correlates with the ability of T cells to secrete IFN-γ in response to α-enolase. PBMC from 12 PDAC patients with and 6 PDAC patients without IgG to α-enolase were left unstimulated or stimulated with α-enolase, and expanded in the presence of IL-2. After 11 days of culture PBMC were tested in a 40 h IFN-γ Elispot in the presence or not of α-enolase. With a cut-off of 10 spots/10^5 cells, eight out of twelve patients that displayed IgG reactivity to α-enolase showed a significantly increased number of IFN-γ secreting cells in response to α-enolase (Fig. 7*a*): in four patients (#5, #6, #7 and #11) this occurred independently of the *in vitro* expansion (Fig. 7*a*, left panel); in four (#2, #4, #8 and #12) IFN-γ production was observed only after *in vitro* expansion (Fig. 7*a*, right panel). None of the six patients who did not display IgG reactivity to α-enolase had IFN-γ secreting cells after the primary

stimulation (Fig. 7*a*, lower left panel) or after the *in vitro* expansion (Fig. 7*a*, lower right panel), and the few spots counted for these patients were much smaller and of lower intensity than those from patients with IgG to α-enolase (data not shown). From patients #12 (with) and #18 (without IgG reactivity) we purified CD4 and CD8 cells prior to the Elispot assay. IFN-γ secreting cells were only present among those from patient #12 and increased after in vitro expansion (Fig. 7*b*).

DISCUSSION

This study demonstrates that α-enolase may be a promising PDAC associated antigen and molecular target suitable for therapeutic approaches. We show that it is abundantly and broadly expressed in both the cytoplasm and cell surface of PDAC. Its surface expression has been reported in myeloid and mammary tumor cell lines [23, 28]. Interestingly, microarray data indicated that α-enolase is not increased in pancreatic tissues from CP, but only in PDAC. This strengthens the hypothesis that up-regulation of α-enolase is associated with pancreatic tumorigenesis rather than general inflammation. In the hypoxia that occurs in tumor sites, α-enolase expression is up-regulated by the presence of an Hypoxia Responsive Element (HRE) in its promoter [29-31]. In addition, α-enolase over-expression has been clinically associated with tumor status for lung and hepatocellular carcinoma [32, 33].

Alpha-enolase induces an antibody response in almost two-third of PDAC patients, but not in healthy donors, non-PDAC tumor patients and chronic pancreatitic patients [18]. PDAC patients did not display signs of autoimmunity, though the presence of anti-α-enolase antibodies has been described in patients with autoimmune disorders [23, 34-39].

It is known that α-enolase expressed at cell surface acts as a plasminogen receptor [28, 40]. The bound plasminogen is more efficiently activated to plasmin and thus contributes to pathological processes such as tumor cell invasion, metastases and inflammatory responses [41]. The role of these antibody responses remains to be defined in the clinical follow-up. Even so, our data show that a clear correlation exists between the presence of antibodies and the ability of PDAC patient T cells to respond to α-enolase. Of note, only an in vitro boost with α-enolase was enough to elicit IFN-γ production by T cells from patients with anti-α-enolase IgG, whereas no T cells from patients without anti-α-enolase IgG, secreted IFN-γ following either primary stimulation or *in vitro* expansion. The induction of an integrated humoral and cellular immune response to TAA has been

proved to efficiently drive clinical responses by tumor patients [42, 43]. Thus, the spontaneous humoral and cellular immune responses elicited by α-enolase in a subset of patients with α-enolase-expressing PDAC may be an highly favourable characteristic for a vaccine target.

Our data demonstrate that α-enolase elicits anti-tumor T cell effectors to kill PDAC cells both in vitro and an in vivo *nu/nu* mouse model, where CF-PAC-1 cell growth was delayed by α-enolase stimulated T cells injected i.v. after tumor was established. Of note, the CTL response to α-enolase spared both autologous DC and normal keratinocytes. Cytotoxicity is dependent on the level of α-enolase expression, which is much higher in PDAC cells than normal cells such as keratinocytes. It will be important to characterize the α-enolase epitopes responsible for T cell reactivity in PDAC to improve their recognition and activation. In this respect, studies with *C. albicans* and *C. tropicalis* have identified cytosolic α-enolase as an immunodominant antigen [44-47], and Sato et al. described a single HLA-DR-restricted peptide of human α-enolase identified in squamous cell carcinoma that elicited cytotoxic activity by CD4 T cells [48]. Our data suggest that α-enolase is recognized in the context of HLA-A2 allele.

Taken as a whole, these data show that a T and B cell integrated anti-tumor response against α-enolase can be induced. It is thus a promising candidate for new immunotherapeutic approaches to be associate with conventional approaches in the treatment of PDAC. Recent data indicated that the combined chemo-immunotherapy may constitute a new strategy with which to control tumor progression [49]. The immune system, indeed, could be elicited in two ways by conventional therapies. Some therapeutic programmes elicit specific cellular responses that render tumor-cell death immunogenic [50]. Other drugs may have side-effects that stimulate the immune system through different mechanisms. Moreover, vaccination against cancer-specific antigens may sensitize a tumor to subsequent chemotherapy [49]. Thus, it may be possible to design adjuvant therapies to elicit anti-α-enolase responses to treat surgically resected patients to avoid recurrent disease, or for untreatable patients to prolong overall survival.

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PC, BT and FN are inventors of a PCT application (WO 2008/037792 A1) entitled “Novel antigens and antibodies associated to pancreatic ductal adenocarcinoma”. Potential investigator conflict of interest has been disclosed to all co-authors.

Figure Legends

Figure 1. Alpha-enolase is differentially expressed in PDAC compared to normal pancreatic tissues. (*a*) Differential expression of the ENO1 (●) and Kras (○) mRNA according to microarray data. The data are shown as base-two logarithm ratio of intensity values compared with median intensity of NP. The p-values are calculated by linear regression analysis: NP vs CP 0.419; NP vs PDAC <0.001; NP vs PaCa44 <0.001; NP vs PDAC xenograft <0.001. (*b*) Mean of normalized intensity relative to α-enolase expression evaluated by WB from 3 PDAC biopsies and 3 adjacent NP. In the insert (upper) one of three representative WB with anti-α enolase mAb from total protein extract from NP and PDAC tissues is shown. Anti-β-tubulin mAb was used as internal control (insert, lower). (*c*) Immunohistochemical analysis of formalin-fixed and paraffin imbedded sections of NP (left panel) and PDAC tissues (right panel) with anti-α-enolase mAb. Stained sections were counterstained with hematoxylin. All images were taken with a x 60 objective. (*d*) CF-PAC-1 and Mia-Pa-Ca-2 cells were seeded on cover glasses, fixed and stained with an isotype control Ig (left panels) or the anti-α enolase mAb (red, right panels). Nuclei are stained in blue by Hoechst 43332.

Figure 2. Alpha-enolase elicits a proliferative response by both CD4 and CD8 T cells.
(*a*) Proliferative response to α-enolase-DC by T cells from 6 healthy donors independently tested. All three T:DC ratios are shown. White bars correspond to T cells cultured with unpulsed DC and black bars to T cells cultured with α-enolase-pulsed DC. The proliferative response was quantitated as ^{3}HTdR uptake. (*b*) T cells were cultured with unpulsed (○), three different concentrations of α-enolase (black symbols) or human albumin (□) pulsed-DC. A mean of three donors independently tested is shown. $*P < 0.05$, values from T cells cultured with α-enolase pulsed DC compared to T cells cultured

with unpulsed DC. (C) Magnetic beads-positively enriched CD4 and CD8 T cells from 4 healthy donors were cultured with autologous unpulsed (white bars) or pulsed with α-enolase (black bars) DC. Results obtained at 20:1 T/DC ratio (10000 DC/well) are shown.

Figure 3. Alpha-enolase induces IFN-γ secretion by T cells. (*a*) DC unpulsed or pulsed with α-enolase were cultured with autologous T cells (called unstimulated T cells, white bars, and α-enolase stimulated T cells, black bars, respectively) for 1 week. Recovered T cells were further stimulated in Elispot plates for 20 h in the presence of α-enolase-DC (left panel), CF-PAC-1 (middle panel) and normal human keratinocytes (hKER) (right panel) previously incubated or not with anti-HLA class I or II mAb. For all stimulation conditions the background (T cells stimulated with medium alone or unpulsed DC) is shown (grey bars). One of three independent experiments is shown. (*b*) CD4 (left panel) and CD8 (right panel) T cells respectively were enriched with magnetic beads from T cells recovered from co-cultures with unpulsed DC or with α-enolase DC, and stimulated with α-enolase or albumin pulsed DC and CF-PAC-1 in Elispot plate. One of three independent experiments is shown.

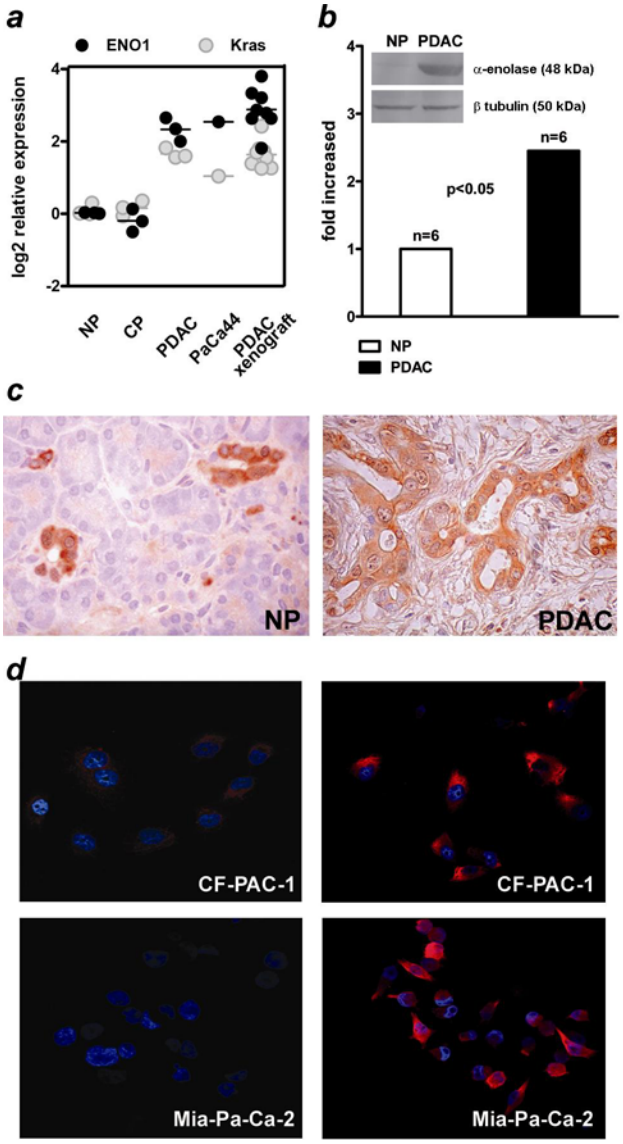
Figure 4. Alpha-enolase induces cytotoxic activity by purified CD8 T cells. Cytotoxic activity by CD8 purified T cells from ustimulated T cells (white symbols) and α-enolase-stimulated T cells (black symbols) was evaluated against HLA-matched CF-PAC-1 (*a*), not matched Mia-Pa-Ca-2 (*b*) autologous DC (*c*) and K562 (*d*) target cells in a 4 h ^{51}Cr release assay. One of six independent experiments is shown. (*e*) Cytotoxic activity against CF-PAC-1 cells by purified CD8 T cells from 5 donors independently tested. Each graph correspond to single donor. All conditions were done in triplicate wells, thus each value of % of lysis is the mean of 3 values. $*P < 0.05$, values for % of specific lysis by CD8 purified T cells from α-enolase-stimulated T cells compared to that by CD8

purified T cells from unstimulated T cells.

Figure 5. Correlation between α-enolase expression and MHC-restricted cytotoxicity. (*a*) The α-enolase fold increased expression was reported by assigning a value of 1 to normalized volume intensity evaluated from the total protein extract of hKER. Cytotoxic activity by T cells from α-enolase-stimulated T cells against HLA-matched hKER, CF-PAC-1 and PANC-1 was evaluated by flow cytometry. Percentage of specific lysis by α-enolase stimulated T cells was calculated by subtracting that by T cells from unstimulated T cells. Anti-HLA class I and II mAb were added to target cells before the incubation with the effector cells. * $P < 0.05$, values for % of specific lysis by α-enolase-stimulated T cells against PDAC cells compared with that against anti-HLA class I mAb pre-treated PDAC cells. (*b*) Alpha-enolase expression analysed in hKER, CF-PAC-1 and PANC-1 cells by WB. One of three representative WB with anti-α enolase mAb on total protein extract from human keratinocytes, CF-PAC-1 and PANC-1 cells is shown. Anti-actin mAb was used as internal control and to normalize volume intensity.

Figure 6. *In vivo* inhibition of PDAC growth by α-enolase specific T cells. (*a*) *Nu/nu* mice were injected with CF-PAC-1 cells s.c. and three days later were challenged i.v. in the absence (□) or presence of unstimulated (◊), albumin- (○) or α-enolase-stimulated (●) T cells. Tumor growth was monitored biweekly. The growth of tumor masses is shown. All *nu/nu* mice were sacrificed when tumor masses reached 15 mm in diameter. ** $P < 0.05$ vs CF-PAC-1 cells alone, plus unstimulated or albumin-stimulated T cells; (*insert*) Hematoxylin staining of formalin-fixed and paraffin imbedded sections of CF-PAC-1 cells 3 days after tumor challenge in *nu/nu* mice. Image was taken with a x 40 objective. Arrow indicates inguinal lymph node.

Figure 7. PDAC patients display an antigen-specific T cell response to α-enolase. (*a*) PBMC from PDAC patients with (upper panels) and without (lower panels) α-enolase specific serum IgG were expanded in vitro for 11 days with low doses of IL-2 in the absence (left panels) or presence (right panels) of recombinant α-enolase. IFN-γ secreting cells were evaluated after further stimulation with recombinant α-enolase in Elispot plates for 40 h. The number of spots in response to α-enolase represented in the graph is obtained by subtracting that in the absence of stimuli. (*b*) CD8 and CD4 T cells were purified after 11 days' culture from patients #12 (with) and #18 (without) α-enolase specific serum IgG and their ability to secrete IFN-γ in response to α-enolase was assessed in the Elispot assay.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β tubulin (50 kDa)
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4
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1
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n=6
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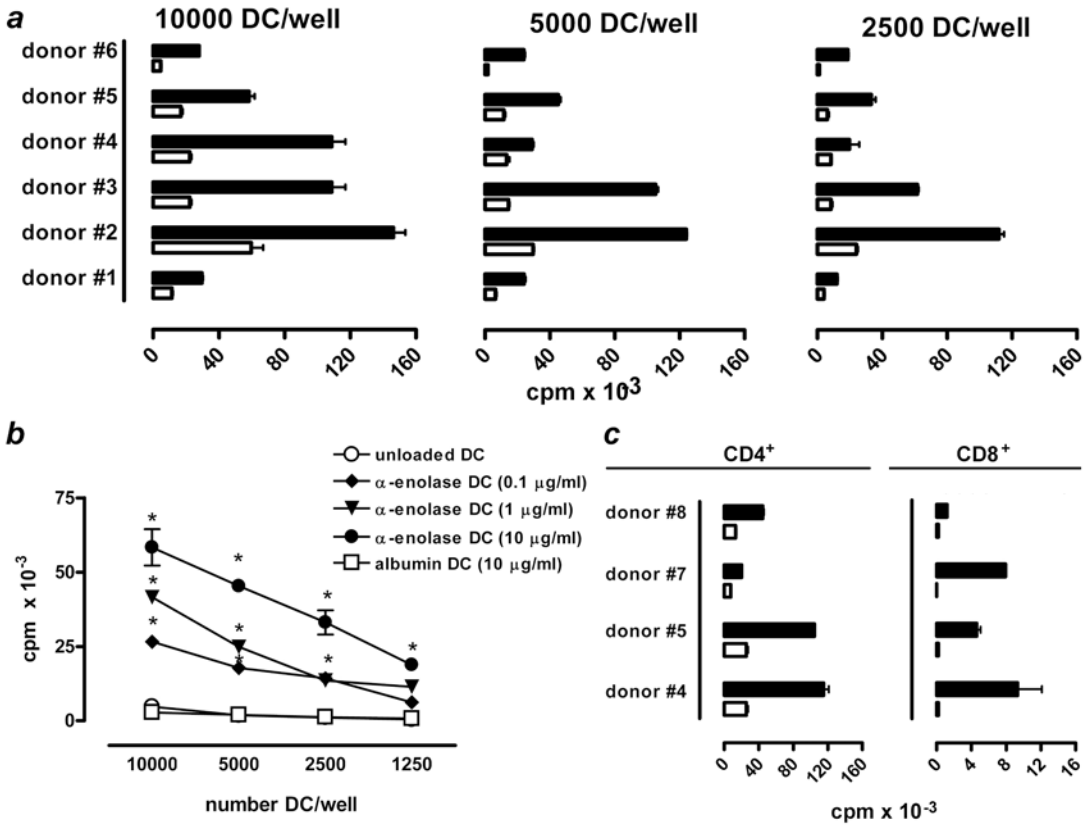
CF-PAC-1
CF-PAC-1
Mia-Pa-Ca-2
Mia-Pa-Ca-2

Figure 1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80
120
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cpm x 10^3
b
unloaded DC
α-enolase DC (0.1 μg/ml)
α-enolase DC (1 μg/ml)
α-enolase DC (10 μg/ml)
albumin DC (10 μg/ml)
cpm x 10^-3
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50
25
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1250
number DC/well
c
CD4+
CD8+
donor #8
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Figure 2

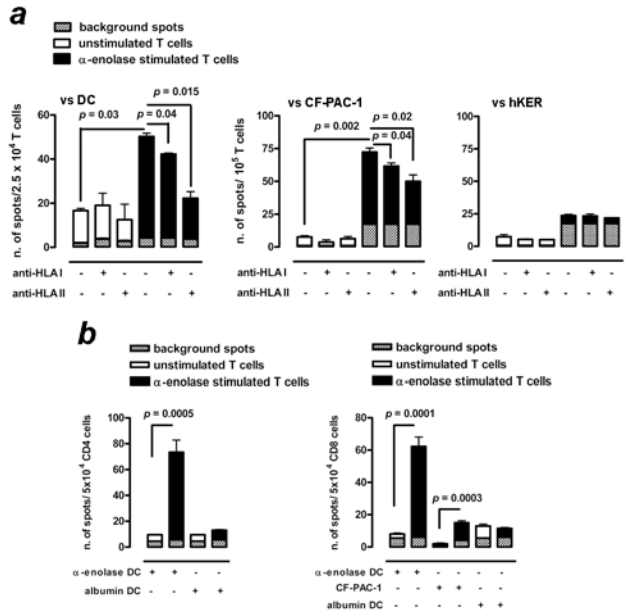
a
background spots
unstimulated T cells
α-enolase stimulated T cells
vs DC
vs CF-PAC-1
vs hKER
n. of spots/2.5 x 10^4 T cells
n. of spots/ 10^5 T cells
p = 0.03
p = 0.04
p = 0.015
p = 0.002
p = 0.02
p = 0.04
anti-HLA I
anti-HLA II
b
background spots
unstimulated T cells
α-enolase stimulated T cells
n. of spots/ 5x10^4 CD4 cells
n. of spots/ 5x10^4 CD8 cells
p = 0.0005
p = 0.0001
p = 0.0003
α-enolase DC
albumin DC
CF-PAC-1

Figure 3

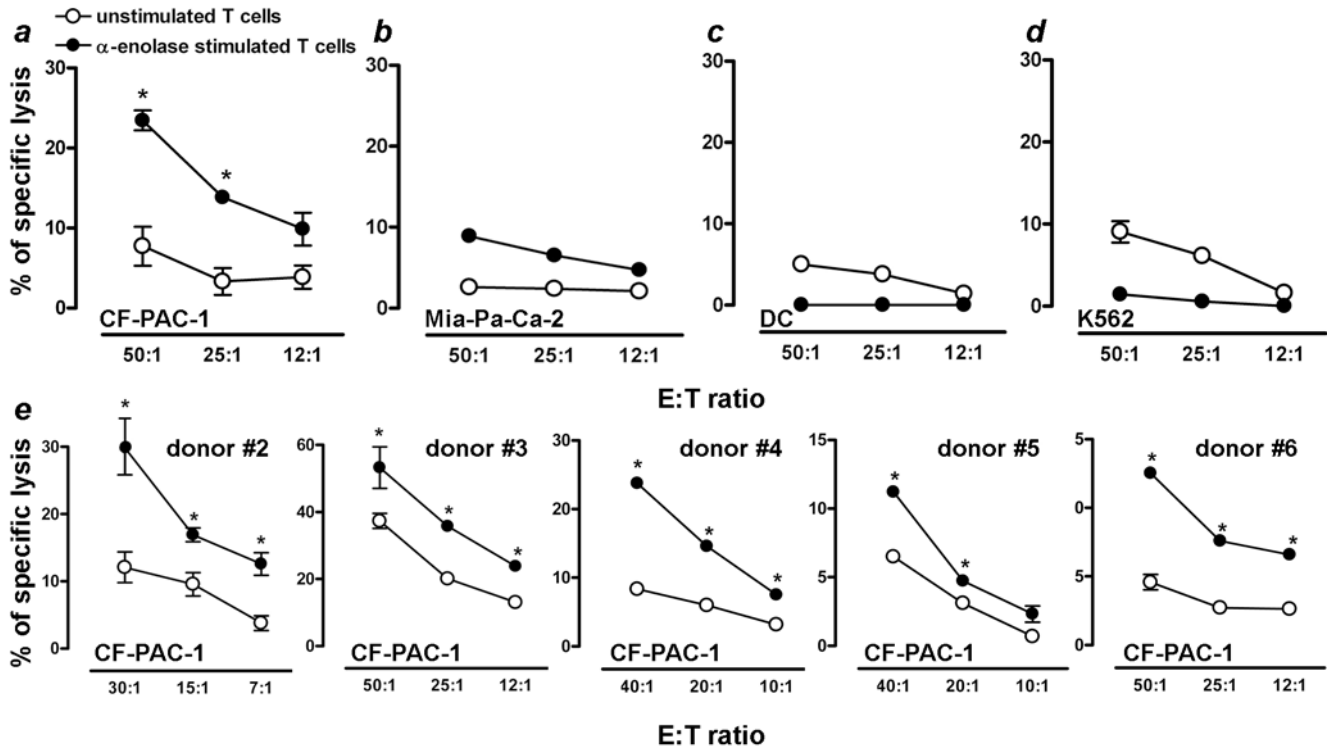
a
unstimulated T cells
α-enolase stimulated T cells
% of specific lysis
CF-PAC-1
50:1 25:1 12:1
b
Mia-Pa-Ca-2
50:1 25:1 12:1
c
DC
50:1 25:1 12:1
d
K562
50:1 25:1 12:1
E:T ratio
e
donor #2
CF-PAC-1
30:1 15:1 7:1
donor #3
CF-PAC-1
50:1 25:1 12:1
donor #4
CF-PAC-1
40:1 20:1 10:1
donor #5
CF-PAC-1
40:1 20:1 10:1
donor #6
CF-PAC-1
50:1 25:1 12:1
E:T ratio

Figure 4

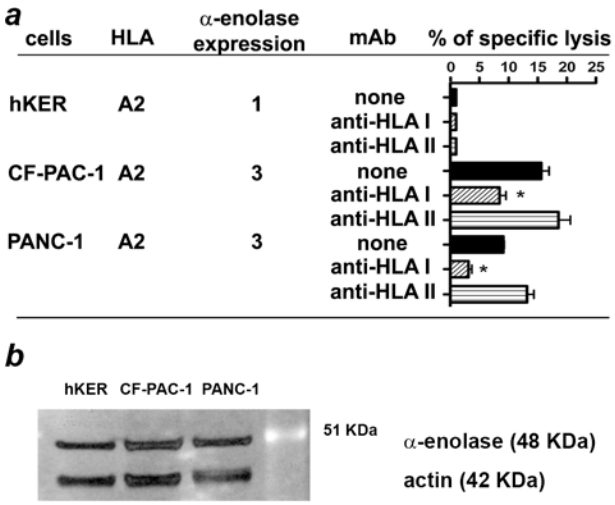
a
cells
HLA
α-enolase expression
mAb
% of specific lysis
0 5 10 15 20 25
hKER
A2
1
none
anti-HLA I
anti-HLA II
CF-PAC-1
A2
3
none
anti-HLA I
anti-HLA II
PANC-1
A2
3
none
anti-HLA I
anti-HLA II
*
*
b
hKER CF-PAC-1 PANC-1
51 KDa
α-enolase (48 KDa)
actin (42 KDa)

Figure 5

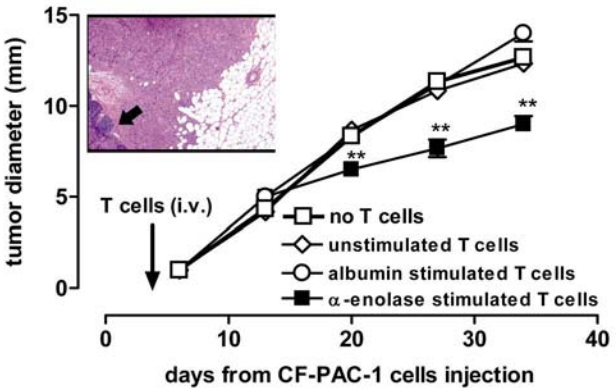
15
10
5
0
tumor diameter (mm)
T cells (i.v.)
no T cells
unstimulated T cells
albumin stimulated T cells
α-enolase stimulated T cells
**
**
**
0
10
20
30
40
days from CF-PAC-1 cells injection

Figure 6

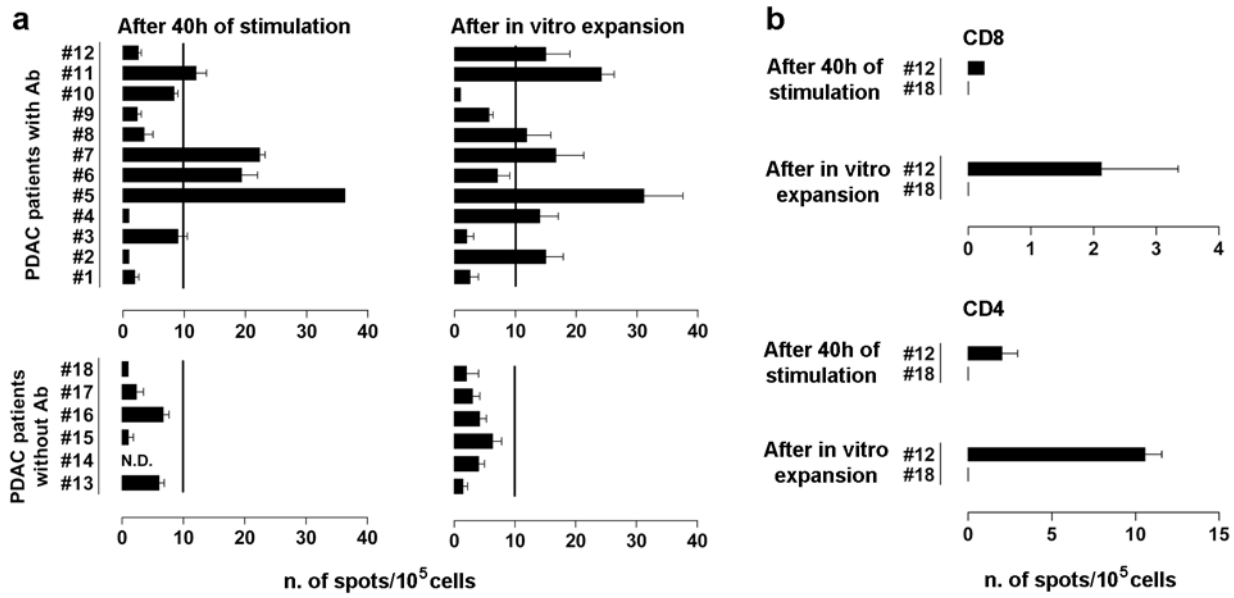
a
After 40h of stimulation
After in vitro expansion
PDAC patients with Ab
#12
#11
#10
#9
#8
#7
#6
#5
#4
#3
#2
#1
PDAC patients without Ab
#18
#17
#16
#15
#14
N.D.
#13
0 10 20 30 40
n. of spots/10^5 cells
b
CD8
After 40h of stimulation
After in vitro expansion
#12
#18
0 1 2 3 4
CD4
0 5 10 15
n. of spots/10^5 cells

Figure 7